Role of Transglutaminase 2 in vascular remodeling
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Chapter 7

General Discussion

Jeroen van den Akker
General Discussion

Although we previously showed that Transglutaminase 2 (TG2) mediates inward remodeling of small arteries, the underlying mechanisms remained elusive. In this thesis, we investigated which of the functions of this pleiotropic enzyme is involved, and how its activity is regulated. In addition, we studied which cell type provides the source of TG2, how TG2 is distributed over intra- and extracellular compartments and which TG2 enzymatic function is responsible for remodeling.

Overview of TG2 in inward vascular remodeling

Figure 7.1 summarizes the major findings on TG2-mediated small artery remodeling, as presented in this thesis. Remodeling starts when smooth muscle cells establish active vasoconstriction for a period of at least several hours. During this period, cell-mediated compaction may displace matrix proteins in an initial process of remodeling that may still be reversible upon vasodilation (Chapter 4). However, if vasoconstriction is maintained, gradual stabilization by transamidation of ECM substrates would occur as a result of the presence and activity of extracellular TG2. Such activity requires a reduction of cytosolic TG2, which triggers translocation to the cell membrane. Reduction and translocation may be facilitated by a relative lack of NO, as occurs under low flow and in hypertension and aging. Extracellular calcium then activates membrane-bound TG2 leading to transamidation of matrix proteins close to the cell membrane (Chapter 6). Such membrane-bound activity would be orchestrated by a complex of TG2, fibronectin and integrins, leading to cross-linking as well as movement of local matrix fibers. Alternatively, transamidation activity at the cell membrane aids in the formation of microparticles (Chapter 5). Possibly, MP secretion occurs preferentially at sites where certain integrins and membrane-bound fibronectin are present. TG2-enriched MPs may function to cross-link the more mature ECM substrates that are not in close proximity to the cell membrane, or act as messenger for other processes leading to cross-linking or synthesis of matrix proteins. In the next sections, the components in this schematic overview are discussed in detail.
Matrix compaction may support inward vascular remodeling

Inward vascular remodeling may be facilitated by cell-mediated compaction of matrix fibers. In previous research, our group had shown that macroscopic compaction of a collagen gel by SMCs was stimulated by exogenous TG2, and could be inhibited by the competitive TG2 substrate cadaverine. Similarly, Eftekhari and colleagues used cystamine to inhibit spontaneous gel compaction. However, the required effective concentration of TG2 and its competitive substrates was quite high. Thus, it remained questionable whether TG2 is indeed required for SMC-mediated tissue compaction. Therefore, in Chapter 4 we tested the hypothesis that SMCs are able to inwardly remodel tissue by TG2-mediated compaction. We showed that single cells displace matrix over a distance of at least 300 μm, but probably much more. We attribute this to cyclic movement of cell protrusions. One could have expected intracellular TG2 effects to play a role in such cellular dynamics and therefore in the matrix displacement. Thus, in Chapter 6, we...
identified several cytoskeletal elements (actin, vimentin, both alpha and beta tubulin, and moesin) as cross-linking substrates in MOVAS cells. Moreover, TG2/eGFP constructs localized to the cell protrusions. However, the rate of compaction was not different between WT and TG2 KO cells. It should be said that this comparison was only made under very basic conditions, using synthetic cells in the absence of extrinsic stimuli or mechanical forces. In vessels, both vasoconstrictors and wall tension are known to increase intracellular calcium, possibly activating intracellular TG2 and changing protrusion dynamics and matrix displacement rate. Another possible explanation for the limited difference in matrix compaction may be that knock-out of TG2 is known to induce significant compensation mechanisms by other members of the Transglutaminase family.

Matrix compaction by pairs and monolayers of cells unexpectedly occurred preferentially perpendicular to the SMC long axis. In Chapter 4 we indicated that, extrapolated to the intact vessel, this would maintain a tightly packed matrix and the development of axial stress while limiting the tendency for inward remodeling, since the latter requires a component of compaction along the SMC long axis. Clearly, this view needs further work.

Altogether, there is little evidence for a role of TG2 in matrix displacement resulting from cycling SMC protrusions. However, in chapter 4 it also became clear that matrix is not only displaced but also actually compacted at a distance from the cell. Moreover, this distant compaction was more reversible in matrix seeded with TG2 KO cells. Thus, these experiments provide evidence for stabilization and local compaction of collagen by released TG2.

**TG2 contributes to remodeling by transamidation**

We previously proposed in our “mold hypothesis” that TG2 contributes to remodeling by cross-linking. Thus, during a period of prolonged vasoconstriction, cross-linking of existing ECM components and synthesis of new elements would provide mechanical stabilization of the vessel at a smaller diameter. Using recombinant TG2 with a mutated active site, we confirmed that isolated vessels could be fixed at a certain diameter by transamidation. We then showed that the in vitro remodeling of vessels by endogenous TG2 was abolished by an active site inhibitor (Chapter 6).

Such experiments using mutated enzymes and active site inhibitors leave little doubt that indeed of all the possible actions of TG2, cross-linking is critical. However, remodeling requires actual displacement and not only cross-linking of the matrix. As indicated above, cyclic movement of cellular protrusions could be involved here. Another possibility, supported by the above distant compaction, is that transamidation of matrix proteins by itself induces matrix shrinkage. When polymers come into close proximity during deformation of a network, cross-linking can lead to local build-up of internal stress. This can cause a mutual pulling
of polymers to each other, as was demonstrated for the cross-linking of actin by filamin. In turn, these deformations may initiate cross-linking of other branches that approached each other. Together, these results support our “mold hypothesis” during inward remodeling where cross-linking is, at least in part, mediated by TG2.

**Transamidation activity is controlled by redox balance**

As any protein rich in sulphydryl groups, TG2 is known to be sensitive to chemical modifications such as disulfide bridging and S-nitrosylation. Indeed, exogenous TG2 required a reducing agent, which reverses these sulphydryl modifications, for sufficient activity leading to remodeling. Considering the fact that no remodeling occurred when this cell-impermeable reducing agent was used in the absence of exogenous TG2, we derived that the pool of extracellular TG2 must be extremely small. However, reduction of the intracellular pool did trigger TG2-dependent remodeling (Chapter 6). Although cells in the vascular wall are traditionally believed to possess a reducing intracellular environment, it has been shown that the redox balance is actually controlled at a subcellular level, and within a subcellular compartment the redox state of proteins can be individually regulated. We believe that the reduction required for TG2 activity must be regulated by a reducing enzyme with high binding affinity for TG2, possibly within a specific subcellular compartment. Indeed, when we incubated remodeled vessels with fluorescein maleimide, there was no obvious difference in the amount of extracellular sulphydryl groups between control, inward and outward remodeled vessels (pilot, data not shown). In Chapter 6, we identified several reducing enzymes that are likely candidates to be involved in TG2 activation.

**Nitric Oxide affects redox regulation of TG2 activity**

Nitric Oxide (NO) is an important signaling molecule in the cardiovascular system. The release of endothelium-derived NO, which causes vasodilation, depends directly on the vascular shear stress level. In addition, nitric oxide in conjunction with ROS plays a key role in the activity regulation of redox-sensitive proteins. Lai et al. showed that TG2 activity can be inhibited by S-nitrosylation of only 1 or 2 out of the possible 18 cysteine groups in TG2. In addition to direct NO-effects, S-nitrosylation renders TG2 more sensitive to GTP inhibition.

In several papers, our group showed that NO plays an important role in the regulation of the direction of remodeling, possibly by inhibition of TG2 activity. In a cannulated vessel model, flow caused a NO-mediated dilation, which inhibited inward remodeling. However, after application of L-NNA, which blocks NO production, these vessels remodeled inwardly. Oppositely, the NO donor nitroprusside inhibited inward remodeling induced by exogenous TG2. In a subsequent paper, a similar NO synthase inhibitor was administered in a
hypertension model. In WT mice, this led to inward remodeling, which was impaired in TG2 KO mice. In Chapter 4 we demonstrated that application of the NO-donor SNAP inhibited the incorporation of a fluorescent TG2 substrate both after intra- and extracellular TG2 activation. Moreover, SNAP fully inhibited the TG2-dependent inward remodeling as induced by the reducing agent DTT. Similarly, Santhanam et al.\textsuperscript{18} administered the NOS-inhibitor L-NAME using an osmotic minipump, and observed increased TG2-mediated cross-linking.

S-nitrosylation/denitrosylation provides a dynamic system of TG2 (in)activation. Interestingly, NO production is impaired in several conditions where TG2 activity is high, such as low blood flow, hypertension, aging and atherosclerosis\textsuperscript{13}. Considering the high sensitivity to S-nitrosylation, it is well possible that even under conditions of low NO production, an active process of denitrosylation is required to induce TG2 activity. This may be accomplished by the enzymes involved in redox balance regulation, as discussed above.

**TG2 activity is mainly present in the medial ECM**

In Chapter 6, we demonstrated that inward remodeling of isolated vessels was associated with TG2 activity in the medial layer, as visualized by incorporation of a fluorescent TG2 substrate. This required the presence of a cell-permeable reducing agent. Incorporation of fluorescent cadaverine occurred especially at the smooth muscle cell membrane and was also elevated in a patchy pattern at the internal elastic lamina. While it may seem that such membrane-bound TG2 activity is much stronger than any activity of (MP-based) released TG2, the latter would lead to a rather diffuse staining that might not have been appreciated in the confocal studies that we performed.

A large array of potential extracellular targets is present near the SMC membrane. However, the pull-down assay, based on cultured SMCs under low reductive stimulation, revealed a small number of specific targets. Fibronectin, which is deposited in the early phase of matrix synthesis, was abundantly cross-linked by TG2\textsuperscript{19}. In addition, substrates such as collagen type I, fibulin-2 and nidogen-1 were identified. Possibly, association of TG2 with specific integrins adds to the specificity for which ECM substrates in the ECM are cross-linked\textsuperscript{4}.

In addition to the cross-linking activity of membrane-bound TG2, there is evidence that this enzyme is involved in fibronectin and collagen fibrillogenesis, a process that occurs at the cell surface for many ECM proteins\textsuperscript{20,21}. The TG2 active site mutant is equally potent as wild-type TG2 in this process, demonstrating that other actions than cross-linking are involved here\textsuperscript{22}. Since membrane-bound TG2 remains associated with fibronectin after polymerization, the fibronectin binding site seems to play a dominant role in this process\textsuperscript{22}. In this thesis we have not further investigated this process, but there may well exist an intimate relation
between formation of new fibrils and cross-linking of more mature ones. Such processes at the cell membrane deserve future attention.

In contrast to the high activity that we observed in specifically the media, several studies revealed a relatively low immunostaining for TG2-specific cross-links in this layer\textsuperscript{5;6;23;24}. This suggests that if transamidation in the media is indeed occurring during inward remodeling, this must be a transient mechanism of tissue stabilization. Possibly, a mechanism exists to break down the TG2 cross-link in order for tissue to be able to continuously adapt to changing mechanical demands. Interestingly, transamidation cross-links can be cleaved by TG2 or FXIII itself\textsuperscript{25}. However, so far the in vivo activators for TG2-catalyzed hydrolysis remain unknown. Contrary to a possible dynamic turnover of TG2 cross-links in the media, it was recently shown that over time TG2 cross-links accumulate in the adventitia (Figure 7.2)\textsuperscript{18}. With aging, the NO supply to adventitial TG2 becomes partly insufficient, causing transamidation and thereby vessel stiffening. These results together present a picture where TG2 affects short-term remodeling by transamidation in the media, while long-term remodeling appears predominantly in the adventitia.

![Figure 7.2: Immunohistochemical localization of TG2 protein and the specific TG2 cross-link in mouse aortic tissue as prepared by Santhanam et al.\textsuperscript{18}: TG-specific cross-links are higher in old compared to young rat aorta, expression is unchanged.](image-url)
**TG2 is transported to the ECM via microparticles**

The passive diameter of blood vessels at higher pressures is believed to be dominated by mature collagen type I and III fibers. A large fraction of these mature fibers are not in close contact with the cell membrane. Their cross-linking and remodeling thus seem to depend on secreted rather than membrane-bound TG2. Our collagen type I gel experiments have indicated that distant compaction indeed can occur. Since TG2 does not possess a signal sequence to the ER/Golgi, TG2 secretion must be alternatively regulated. We hypothesized that TG2 secretion occurs via the formation of microparticles (MPs).

Currently, little is known about the mechanisms of MP formation and secretion. In a recent review Burnier et al.²⁶ discriminated between 2 mechanisms of MP formation. Ectosomes are formed via outward blebbing of the cell membrane. Proteins that were originally at the inside of the cell membrane may end up at the outside of the MP after flipping of membrane phospholipids. Alternatively, endosomes result from inward blebbing, which causes intracellular membrane proteins to end up at the outside of the MP. Multiple endosomes are then collected in a multi-vesicular body before they are released from the cell as exosomes²⁶. Considering the large size of MPs (or multi-vesicular bodies!) enriched in TG2/eGFP that we observed by immunofluorescent microscopy (Chapter 5), this would speculatively plead for a secretion of TG2 via the endosome-exosome pathway.

In addition to being transported by MPs, TG2 may play an active role in MP formation. Indeed, in Chapter 5 we demonstrated that transamidation activity stimulated MP formation. Cytoskeletal elements undoubtedly play a role in MP formation, notably via the endosome/exosome mechanism, and as indicated above many of these elements are targets of TG2. We tested whether reducing agents activate MP formation via activation of TG2. However, stimulation of TG2/eGFP-transfected smooth muscle cells with the reducing agent DTT did not increase the fraction of eGFP-positive microparticles, nor did it increase the absolute number of MPs (pilot, data not shown). These preliminary data suggest that reduction of intracellular TG2 by itself is not sufficient to stimulate TG2 release via increased MP production. Possibly, the release into the matrix depends on interaction with extracellular fibronectin, since it is previously shown that the fibronectin-binding site in TG2 is indispensible for TG2 secretion²⁷. This would also help to explain the high level of cross-linking that we observed in fibronectin (Chapter 6). Moreover, secretion may occur in conjunction with integrins α5β1 and ανβ3 that frequently colocalize with concentrated spots of TG2²⁸,²⁹.

Altogether, TG2 appears to be secreted into the extracellular space via microparticles. We suggest that an initial reduction step is required for translocation to the cell membrane and cross-linking activity for formation of MPs. MPs may transport TG2 over long distances, but also increase stability of...
extracellular TG2, which is a known target for proteases such as MMP-2\textsuperscript{30-32}. Based on the current data, we speculate that transamidation of maturated extracellular substrates occurs via MPs enriched in TG2. This may be the case not only for proteins in the media, but for the adventitia as well, considering the massive presence of collagen and sparse cellular density.

Unresolved issues and recommendations for future direction

The work in this thesis addressed the role of TG2 in inward vascular remodeling, focusing on issues such as the cellular secretion and extracellular activity. Thus, the role of TG2 in vascular events such as atherosclerosis, vessel permeability and angiogenesis\textsuperscript{33;34} was not considered. In addition, other factors influencing remodeling, such as the facilitation by inflammation\textsuperscript{35}, are beyond the scope of this thesis. This section discusses several issues that remained unresolved and includes some recommendations for future research.

Since inward remodeling is dependent on both vessel tone\textsuperscript{3;35} and TG2\textsuperscript{1}, the large pool of TG2 in smooth muscle cells is likely to be involved. We previously showed that prolonged vasoconstriction, but not passive collapse to a small diameter, is necessary to induce inward remodeling\textsuperscript{2;3}. An attractive possibility is that tone activates TG2, either by affecting the redox state, the secretion of MPs or otherwise. The other way around, it has been speculated that TG2 directly or indirectly stimulates the level of tone\textsuperscript{36}. TG2 is known to play a role in the signal transduction pathway activated by α₁-adrenoreceptors\textsuperscript{37}. However, when we tested α₁-adrenergic signaling in mesenteric arteries by measuring the contractile response to phenylephrine, this showed no differences between vessels obtained from wild-type or TG2 KO mice\textsuperscript{6}. Therefore, we preliminary concluded that TG2 does not mediate inward remodeling of these small arteries by stimulating α₁-adrenergic signaling. In addition, TG2 can stimulate vasoconstriction through the RhoA/ROCK-2 cascade\textsuperscript{38;39}. TG2 can trigger RhoA signaling either via transamidation to serotonin\textsuperscript{40} or various other polyamines\textsuperscript{41}, or via integrin clustering when cells adhere to substrates like FN via membrane-bound TG2\textsuperscript{42}. Indeed, maximal constriction of aortic vessel segments upon either high potassium or serotonin stimulation was significantly inhibited both by cystamine and dansylcadaverine\textsuperscript{24}. In addition, angiotensin-II mediated constriction of mesenteric arterioles was inhibited by these TG2 competitive substrates\textsuperscript{43}. Therefore, the question whether TG2 actively contributes to vessel tone remains to be determined. This is especially of relevance since our group previously showed that Rho kinase was required to maintain a basal tone level in isolated mesenteric arteries\textsuperscript{44}.

In addition to a role for TG2 in SMC contractility, it needs to be tested whether contractile SMCs are able to compact matrices perpendicular to their long axis orientation, just as the synthetic SMCs in Chapter 4. TG2 may play a more
pronounced role in compaction in these contractile cells, because they might accommodate the required calcium influx for transamidation of cell-protrusion elements. These experiments should also be performed in models that more closely resemble the vessel wall.

Although we showed that reduction of TG2 is a critical step for activation (Chapter 6), the nature of the group to be reduced remains to be determined. This can be a thiol modified by either disulfide bridging, S-nitrosylation or S-glutathiolation. Immuno electron microscopy of thiols and TG2 could form an approach here. In addition, the physiological enzyme responsible for reduction of TG2 deserves further attention. Here, the list of candidates retrieved from our gene array approach can function as a starting point.

The in vivo substrates for transamidation need to be identified further. In particular, our pull-down results need to be confirmed at the vessel level during in vivo or in vitro remodeling. The single cell compaction setup presented in Chapters 3-4, combined with fluorescent TG2, could then be used to study the interaction of TG2 with such substrates. Moreover, the local mechanical effect of transamidation of matrix proteins can be assessed with active microrheology using our optical tweezer setup. In such a system, a particle is manipulated at the nano scale with an optical trap. The visco-elastic properties of the surrounding matrix are then derived from the dynamic displacements nearby trapped particles using laser interferometry.

While the concept of tissue stabilization by transamidation is widely accepted, it remains to be determined how these cross-links can be broken down in vivo. This is indispensible in order to achieve a dynamic system that retains its capability for tissue remodeling over time.

Further insight into the mechanism of TG2 secretion is critical, since knowledge on the nature of this pathway facilitates research on the triggers for TG2 activation and externalization. Chapter 5 showed that after transfection of TG2/eGFP into SMCs, the eGFP signal could be detected in microparticles. However, the undisputable presence of TG2 in these MPs remains to be proven. Unfortunately, preliminary attempts with antibodies in both western blots and flow cytometry were unsuccessful. An indication for an active contribution of TG2 in MP formation can be obtained by comparing the MPs secreted from WT and TG2 KO erythrocytes, where compensation by other TG family members is limited. In addition, application of cell-permeable inhibitors of transamidation is expected to decrease the number of secreted MPs. Similarly, MP formation is likely to be inhibited upon increasing the level of intracellular NO, thereby providing a possible physiological switch. If TG2 is indeed secreted via MPs, the question remains whether this TG2 is inactive inside the MP, or active at the MP membrane. This should be tested in vitro under conditions that leave the MP integrity intact.
Clinical Implications

Since TG2 is an ubiquitously expressed enzyme, a simple inhibition of its cross-linking activity in an attempt to improve small artery caliber in hypertension and other cardiovascular diseases will not be without side effects. This is especially the case since most, if not all, current active site inhibitors also act on the coagulation Factor XIII due to the high sequence homology with TG2\textsuperscript{47}. In addition, TG2 is not necessarily a ‘bad’ enzyme for the vascular system. Our studies point at a role in the maintenance of a normal organization, while also plaque stability may depend on transamidation\textsuperscript{48}. Any attempt for the therapeutic interference with TG2-related processes therefore needs to attack specific cells and conditions. The inhibiting role of NO on transamidation would provide a starting point\textsuperscript{18}. In addition, interfering with specific redox reactions and with MP formation could have therapeutic potential. However, clearly, much more basal research will be needed for this step can be taken.

We studied very fundamental processes related to matrix organization in the context of small artery remodeling. It seems very likely that such processes are of relevance for a wide range of other cardiovascular and non-cardiovascular pathologies. Thus, the redox dependence, microparticle formation, membrane bound and distal effects that we described may be involved in the role of TG2 in vessel stiffening associated with aging, stabilization of atherosclerotic plaques or vascular injury, and calcification. Moreover, extracellular transamidation affects a number of other pathologies, among which are several types of cancer and neurodegenerative diseases\textsuperscript{47}.

Conclusions

We found Transglutaminase 2 to be an intriguing enzyme, whose role in vascular remodeling is only beginning to emerge. This enzyme is not easily giving away its secrets, and the current thesis can only be a small step. The new mechanisms that we identified warrant further research and should, in the future, provide new therapeutic tools for hypertension and ischemic diseases, as well as for a range of other pathologies.
References


